FREEZING PRETREATMENT OF LIGNOCELLULOSIC MATERIAL: A CHEAP ALTERNATIVE FOR NORDIC COUNTRIES

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Abstract
Using lignocellulosic biomass as an alternative transportation fuel resource is attractive due to its abundance and low cost. Conventional pretreatment methods such as steam explosion or ammonia fibre expansion need technically complex devices, high energy input and the use of toxic chemicals in order to achieve high glucose and ethanol yields. In this paper freezing pretreatment of barley straw was investigated as a low energy input and cost effective alternative pretreatment method for second generation bioethanol production. Laboratory and field tests were conducted. In the freezing pretreatment method milled biomass mixed with water was frozen up to -18°C for a certain period of time. The frozen biomass was then defrosted to room temperature (ca. 22°C). The freezing cycles were repeated several times to study the effect of repeated freezing. In addition, field experiments were carried out by taking samples of barley straw that was stored outside both in bales and swaths during a time period from September to March of two consecutive years (in winter 2014 to 2015 and 2015 to 2016). Freezing pretreatment was followed by enzymatic hydrolysis and fermentation. Glucose and ethanol concentrations were used as indicators of pretreatment efficiency. The highest hydrolysis efficiency of 30.37% was achieved in the laboratory tests where biomass was frozen and thawed six times. The best result from a field test was 2.56% from straw stored as swath and gathered in March. Fermentation yields ranged up to 66.37 g/kg. Field tests show that in the bale the temperature never fell below freezing and thus, the pretreatment was not effective. In the swath the straw does freeze through but the winter in Estonia was too mild for this method to work effectively.

Keywords: bioethanol, lignocellulosic biomass, biofuel, freezing pre-treatment.

INTRODUCTION
Lignocellulosic biomass has been widely used as an energy source for a long time. Most commonly it is used for heat and electricity production by burning it directly. Yet, most of the primary energy (up to 80%) consumed worldwide today is produced from fossil fuels. From this 58% is used for the transportation sector alone [1]. Demand for energy rises daily and therefore, new, cheap and sustainable alternatives for liquid fuel production are needed. At the moment biofuels are considered to be the most favourable choice due to their abundance, renewability, biodegradability and cost-effectiveness [1–3]. Global bioethanol production in 2015 was 98.3 billion litres, majority of which was first generation bioethanol, produced from food crops such as sugar cane, maize, etc. [4]. Thus, a new and cheap alternative has to be found to prevent food crops usage as fuel production feedstock. Plants consist primarily of plant cell walls from which about 75% are different polysaccharides, which can be used for ethanol production with right conversion treatment [5]. Therefore, lignocellulosic
Biomass as a potential ethanol production feedstock has attracted the attention of many researchers worldwide. Ethanol production from lignocellulosic biomass consists of four main steps: pretreatment, hydrolysis, fermentation and distillation [3]. The hydrolysis, fermentation and distillation processes have already been widely studied and optimal methods have been found. However, optimal and cost-effective pretreatment process is yet to be found.

Although several commercial lignocellulosic ethanol production plants have started to work worldwide, second generation bioethanol is still not preferred due to its high cost. The high cost is caused by the complexity of pretreatment process. During the pretreatment the biomass structure has to be broken down to ensure better access for enzymes to hemicellulose and cellulose fibres [6]. Today, most commonly used methods for opening biomass cellular structure for enzymes are steam explosion, ammonia fibre explosion (AFEX), SO$_2$ and CO$_2$ explosion [7]. These methods result in high sugar and ethanol yields. Yet all of these methods are energy-intensive or use chemicals, which are expensive and a potential threat to the environment.

In this paper the efficiency of freezing pretreatment method was investigated on barley straw as a cheap pretreatment method for Nordic countries. In addition, it was investigated whether Estonian winter would be suitable for implementing such pretreatment method.

**MATERIALS AND METHODS**

**Biomass**
The barley straw was used as feedstock since it is one of the common crops grown in Estonia with relatively high cellulose content. Samples were gathered once a month from both swaths and bales that were stored outside. The experiments with bales were conducted during winter 2014-2015 and with swaths during the autumn and winter period from September 2015 to March 2016. Biomass collected in September was used in laboratory pretreatment experiments.

**Pretreatment**
Cellular structure of the biomass was broken down using two different approaches. For the laboratory tests milled and moistened biomass was frozen in a freezer. For the field tests barley straw was left in swaths and bales on the field to freeze.

In the laboratory tests 100 g of milled dry biomass was moistened with distilled water. The biomass was inserted into a closed thermo-box and placed into a freezer at a temperature of -18°C. Only one parameter, the repetition of 24 hour freezing cycles was changed during the experiments. One freezing cycle included inserting the sample in a freezer for 20-24 hours following a melting of the frozen sample at room temperature. 7 different experiments in total were conducted. One for the determination of reference point with initial biomass and 6 experiments with different 24 hour freezing cycles from freezing 1 to 6 times. All experiments were done at least in triplicate.

The field experiments took place during winters 2014-2015 (straw bales) and 2015-2016 (the barley straw stored in swaths).

During the winter 2014-2015 several straw bales were left on the field. For the reference point first sample was taken right after baling the straw in August 2014. The following samples were taken 30, 60 and 90 days after baling the straw.

In order to get a reference point for the swaths the first samples were taken in September 2015 right after harvesting. During October and November no samplings were taken due to the warm weather. The average temperatures were 13.3°C in September, 5.8°C in October and 4.9°C in December. In these three months there were only a few night frosts (minimum temperature measured during these three months was $T_{\text{min}} = -8.8°C$) [8]. Further averaged samples from bales and swaths were taken after first decent night frosts, where temperature fell below -8°C, in December, and from thence once a month until March.
The samples were thawed and dried at room temperature to a moisture content less than 10%, and milled using Retch SM 100 mill (Retch GmbH) to a particle size of 1 mm or less. At least 3 parallel experiments were performed with all of the samples.

Hydrolysis
The pretreatment was followed by an enzymatic hydrolysis. 100 g of dried and milled biomass was mixed with distilled water in order to amount the total volume of mixture to 970 mL. The hydrolysis was carried out using the enzyme complex Accelerase 1500. The enzyme was used in ratio of 0.3 mL per g of biomass, bringing the total volume of the mixture to 1000mL. The hydrolysis was carried out in 1000 mL flasks at constant mixing in a shaker-incubator Unimax 1010 at a rotational speed of 250 rpm and at the temperature of 50ºC for 24 hours. The glucose concentrations were measured after hydrolysis.

Fermentation
The fermentation was carried out in 1,000 mL flasks under low oxygen conditions achieved by sealing flasks with fermentation tubes. In order to initiate the fermentation process 2.5 g of dry yeast *Saccharomyces cerevisiae* per 100 g of biomass was added. The process lasted for 7 days after which ethanol concentrations in the samples were measured.

Analysis
The cellulose, hemicellulose and lignin content in the biomass were determined in the Estonian University of Life Sciences in the Laboratory of Plant Biochemistry according to AOAC 973.1 (Association of Official Analytical Chemists) standard. The glucose and ethanol yields were determined using electrochemical analyser Analox GL6.

RESULTS AND DISCUSSION

Biomass analysis
The suitability of the use of biomass for ethanol production can be characterized on the basis of its relative cellulose, hemicellulose and lignin content. Previous research has shown that cellulose content and ethanol yield per kg of biomass are directly interrelated [8]. In this research, the barley straw was investigated due to its relatively high cellulose content and also because it is an agricultural waste product, which makes it a suitable biomass for bioethanol production. The swath samples harvested in September 2015 had the highest cellulose and hemicellulose content of 45.74% and 28.96%, respectively, and the lowest lignin content of 7.77%, while the samples gathered in December 2016 had the lowest cellulose and the highest lignin content of 41.86% and 9.47%, respectively.

The initial cellulose relative content slight decrease during time period of September to December 2015, was due to the warm (T > 0ºC) and rainy weather conditions which allowed fungi and bacteria to degrade cellulose. In December came the first longer lasting (t ≥ 24h) frosts (T < -10ºC), which halted the vital activity of the fungi and bacteria and degraded hemicellulose, therefore, increasing the relative cellulose content in biomass indirectly. The results of biomass analysis conducted in the Plant Biochemistry Laboratory of the Estonian University of Life Sciences are shown in Table 1.

<table>
<thead>
<tr>
<th></th>
<th>Cellulose</th>
<th>Lignin</th>
<th>Hemi-cellulose</th>
</tr>
</thead>
<tbody>
<tr>
<td>Swath</td>
<td></td>
<td></td>
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<tr>
<td>At harvest</td>
<td>45.57</td>
<td>7.77</td>
<td>28.96</td>
</tr>
<tr>
<td>3 months</td>
<td>41.86</td>
<td>9.47</td>
<td>28.26</td>
</tr>
<tr>
<td>4 months</td>
<td>44.73</td>
<td>9.19</td>
<td>28.49</td>
</tr>
<tr>
<td>5 months</td>
<td>43.98</td>
<td>9.35</td>
<td>28.35</td>
</tr>
<tr>
<td>6 months</td>
<td>44.18</td>
<td>9.30</td>
<td>26.97</td>
</tr>
<tr>
<td>Bales</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>At harvest</td>
<td>39.76</td>
<td>5.70</td>
<td>30.51</td>
</tr>
<tr>
<td>1 month</td>
<td>38.63</td>
<td>6.62</td>
<td>29.55</td>
</tr>
<tr>
<td>2 months</td>
<td>41.47</td>
<td>7.37</td>
<td>28.57</td>
</tr>
<tr>
<td>3 months</td>
<td>39.55</td>
<td>7.08</td>
<td>28.96</td>
</tr>
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The fibre analysis showed that even though the straw was stored outside and the weather conditions were not ideal no significant loss in cellulose content was noticed.

**Effect of freezing pretreatment**

**Freezing pretreatment: in laboratory**

The change in glucose yield with freezing the biomass in laboratory is illustrated in Figure 1.

![Figure 1. Change in glucose and ethanol yield gained with different freezing repetitions in laboratory.](image)

The highest cellulose to glucose conversion rate of 116.97 g per kg was achieved at laboratory experiment with freezing and thawing soaked biomass for 6 times with an efficiency of 25.58% while the lowest result was gained with freezing and thawing the biomass for four times. Still, the conversion rate and efficiency for the lowest result were 64.77 g per kg and 14.16%, respectively, which still exceeded the best field experiment result 4.56 times.

A decline in glucose yield can be seen with freezing and thawing biomass 3 and 4 times. Since every herbaceous species has different characteristics, an individually suitable pretreatment method has to be determined and adopted to it [9]. The efficiency of hydrolysis depends greatly on specific plants maturity, also. The more matured the plant the higher lignin content it has [10], thus making the access to cellulose for enzymes much more difficult. So, the decrease in glucose yield may be caused by the proportion of more different herbaceous materials in the sample. Although there was a decrease in glucose yield, the ethanol yield still increased slightly and fermentation efficiencies exceeded the limit of 100%. The highest fermentation efficiency rated was 139.59%. This means that 24 hour hydrolysis process was not enough for hydrolysing all liberated cellulose and the conversion process continued during the fermentation process. This indicates that the sample contained significant amount of some other herbaceous or woody biomass with higher lignin content than the barley straw.

Based on the results gained with the Analox GL6 analyser ethanol yield in g per kg of biomass was calculated. The effect of freezing pretreatment (field and laboratory experiments) to ethanol production process was estimated based on the calculated ethanol yields.

For both laboratory and field experiments yeast *Saccharomyces cerevisiae* was used after hydrolysis to convert liberated sugars into ethanol.

Although the glucose yield was intermittent the ethanol yield increased steadily in accordance with the increase in freezing and thawing repetitions. The best result was gained with 6 freezing repetitions resulting in 66.37 g of ethanol per kg of straw.

The increase in ethanol yield with increasing freezing and thawing repetitions was expected. During the freezing the moisture in the cells increases its volume and breaks the cell structure. With every freezing repetition the initial cell structure was more and more damaged, which enabled to expose the cellulose to the enzymes in the following process steps.

**Freezing pretreatment: in field**

To verify the conclusions made on the laboratory experiments field experiments were conducted at two consecutive winters. In one case with the barley straw stored in bales and in other case with the barley straw stored in swaths.

First field experiments with the freezing pretreatment were performed during winter.
2014-2015 with the barley straw stored in bales. The experiments showed that the temperature in the barley straw bales never fell below 0°C (only thin top layer froze) and therefore no alteration in the cellular structure was detected. No significant change in glucose and ethanol yields was detected also. The glucose yields varied from 11.8 to 12.6 g per kg of biomass and ethanol yields from 19.76 to 21.87 g per kg of biomass. Since the results did not differ significantly from each other therefore no further investigation was conducted.

Second field experiments were conducted with the barley straw stored in swaths. Figure 2 illustrates the change in glucose yields for field experiments conducted with the barley straw stored in swath in winter 2015 and 2016.

![Figure 2](image)

**Figure 2.** Change in glucose and ethanol yield after hydrolysis and fermentation of straw gathered from swath.

The best field test from the barley straw stored in swath resulted in a conversion efficiency of 2.56% and a conversion rate of 11.32 g per kg. It was achieved with straw gathered from swath in March 2016. The lowest glucose yield was gained from straw gathered in December 2015. The hydrolysis conversion rate was 4.53 g per kg with the efficiency of 1.08%. Figure 2 shows a similar steady growth trend as was seen with the laboratory experiments on Figure 1. The ethanol yield increased for almost the entire duration of the test series except from the result of straw gathered in February. The only difference with laboratory experiments was the order of magnitude, which was the result of poor weather conditions.

The highest ethanol yield gained was 5.40 g per kg of biomass from the straw gathered in March 2016 as it was expected due to the fact that it was subjected to the most freezing and thawing repetitions. The lowest result was 1.68 g per kg of biomass from straw gathered in December 2015. The fermentation efficiencies ranged from 70.35% with the straw gathered in January to 118.10% with the straw gathered right after harvesting. The highest result for the sample gathered in December was expected due to the fact that it had the lowest cellulose content of 41.86%.

The fermentation process efficiency quite frequently exceeds 100%, yet hydrolysis process efficiencies hardly exceed 30%. This indicates that 24 hour hydrolysis was quite often not enough to hydrolyse all liberated cellulose and process endured during the fermentation process.

The field experiments confirmed the conclusion made on the results of the laboratory tests, that the more freezing repetitions the higher ethanol yields will be gained.

**CONCLUSIONS**

The fibre analysis showed that the cellulose content in the biomass did not decrease significantly during the winter.

Initial laboratory experiments with freezing pretreatment showed potential as a cheap and quite effective pretreatment method for Nordic countries. The glucose and ethanol yields gained varied from 64.67 to 138.90 g per kg and from 36.20 to 66.37 g per kg and with the efficiencies of up to 30.37% and up to 139.59%, respectively. The best results were gained with freezing and thawing the biomass 6 times as it was expected. Yet, due to the poor weather conditions field experiments did not confirm the initial assumptions. The best field experiment
resulted 5.40 g per kg in glucose yield and 2.56 g per kg in ethanol yield. For the straw stored in bales the cold weather had no significant effect on the glucose or ethanol conversion processes due to the fact that the temperature in the bale never fell below freezing point (T˃0°C). The glucose and ethanol yields varied from 11.8 to 12.6 g per kg and from 19.76 to 21.87 g per kg of biomass, respectively. The results of the field experiments confirm that even though high cellulose to glucose conversion efficiencies were not achieved, still alteration in the biomass structure was detected. Therefore it can be considered as a pre-pretreatment method for some other pretreatment method, such as nitrogen explosion, AFEX, steam explosion, etc. in order to lower production costs, by increasing glucose and ethanol yields, with the same amount energy input.

REFERENCES